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Transcriptional Regulation of Fatty Acid Biosynthesis in *Lactococcus lactis*

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Here we study the influence of the putative fatty acid biosynthesis (FAB) regulator FabT (originally called RmaG [*Llmg_1788*]) on gene transcription in *Lactococcus lactis* MG1363. A strain with a knockout mutation of the putative regulator was constructed, and its transcriptome was compared to that of the wild-type strain. Almost all FAB genes were significantly upregulated in the knockout. Using electrophoretic mobility shift assays (EMSAs) and DNase I footprinting, the binding motif of the regulator and the binding locations in the genome were characterized. Fatty acid composition analysis revealed that a strain lacking FabT contained significantly more saturated acyl chains in its phospholipids. This observation demonstrates that the vital pathway of FAB in *L. lactis* is regulated by the repressor FabT.

Membrane phospholipids are essential for life; they contain a hydrophilic head group and two hydrophobic tails esterified to a glycerol moiety. The hydrophobic tail is usually composed of a stretch of hydrocarbons denoted as acyl chains. Biosynthesis of saturated fatty acids (SFA) in bacteria is performed by multiple conserved enzymes in a multistep process. Based on the sequence similarity of genes in the *fab* regulon, we describe here the most likely fatty acid biosynthesis (FAB) route in *Lactococcus lactis*. The acetyl coenzyme A (acetyl-CoA) carboxylase (ACC) complex, consisting of AccABCD, catalyzes an acetyl-CoA-into-malonyl-CoA conversion (1). The CoA is replaced by an acyl-carrier protein (ACP) by FabD, a malonyl-CoA:ACP transacylase (2). Fatty acid elongation rounds are initiated by FabH (β-ketoacyl-ACP synthase III) by condensing an acetyl-CoA with malonyl-ACP (3). The first reductive step in the FAB elongation is performed by β-ketoacyl-ACP reductase (FabG) producing β-ketoacyl-ACP and $NADP^-$ (4). This β-ketoacyl-ACP is dehydrated by FabZ (β-hydroxyacyl-ACP dehydratase), resulting in a *trans*-2-enoyl-ACP (5, 6). The final step in lactococcal FAB elongation is a second reduction step executed by *trans*-2-enoyl-ACP reductase I FabI, giving an acyl-ACP (7, 8). Further elongation rounds start by the condensation enzyme FabF β-ketoacyl-ACP synthase II through the addition of an acyl group from malonyl-ACP (9, 10). The resulting β-ketoacyl-ACP can continue through the elongation cycle by reduction by FabG again. For *L. lactis*, no enzymes that can process the acyl-ACP into phospholipids are identified. The only protein is PlsX, annotated in *L. lactis* as a putative acyltransferase. Investigations on PlsX from *Bacillus subtilis* showed that the enzyme is able to form acylphosphate from acyl-ACP (11, 12).

FAB has been shown to be a coordinated process in the model organisms *Escherichia coli* and *B. subtilis*, in which FAB is under tight control of the transcriptional regulators FadR/FabR and FapR, respectively (13). The bifunctional *E. coli* FadR activates the essential gene *fabA* (14). When sufficient amounts of long-chain acyl-CoA have been produced, some of these molecules bind to FadR, which results in derepression of the fatty acid degradation pathway (β-oxidation) specified by the *fad* operon (15). FabR is the transcriptional repressor of *fabA* and *fabB*, two genes that are required for the synthesis of unsaturated fatty acids (UFA). FabR represses FAB and the first steps of phospholipid synthesis in *E. coli* (16). The *B. subtilis* FapR regulator functions as a malonyl-CoA sensor, whereby complex formation of FapR and its corepressor malonyl-CoA results in the repression of the transcription of the FAB genes (17).

Regulation of FAB in *Lactococcus lactis* is poorly understood; it is important to understand this regulation, though, in view of the possible involvement of FAB in flavor formation pathways in this industrially relevant microorganism. Because of the synteny of their *fab* gene clusters, *L. lactis*, *Enterococcus faecalis*, and *Streptococcus pneumoniae* were grouped together (18). The regulator of FAB in *S. pneumoniae* and *E. faecalis* is FabT. In *S. pneumoniae*, this protein binds to the upstream region of *fabK* and to that of *fabT* itself and is corepressed by the acyl carrier protein (ACP) coupled to C16:0 and C18:0 acyl chains (19). There seem to be only two binding sites for the regulator FabT in the *fab* gene cluster. The *L. lactis* operon carries more and larger intergenic spaces where a regulator can possibly bind. A similar situation occurs in *E. faecalis*, in which FabT is capable of binding to three regions upstream of the genes *fabT*, *fabK*, and *fabI/fabF1*. In *L. lactis*, two *fab* genes possess a paralog outside the operon, i.e., *fabZ* (outside, *llmg_0538/fabZ1*; inside, *llmg_1781/fabZ2*) and *fabG* (outside, *llmg_1760/fabG2*; inside, *llmg_1784/fabG1*). Special attention should be given to *fabZ1* since it shares an upstream region with the enoyl-ACP reductase gene *fabI*, an essential part of FAB. In *S. pneumoniae* and *E. faecalis*, *fabI* is present in the *fab* cluster, where it is named *fabK*. *E. faecalis* and *L. lactis* both contain *fab* genes on two locations on the chromosome. The remainder of the cluster, the genes for the acetyl-CoA carboxylases *accABCD*, the acyl carrier protein gene *acp*, and the FAB genes *fabDFGHZ* are present in *L. lactis*, *E. faecalis*, and *S. pneumoniae* with similar synteny (Fig. 1A). In addition, all genes of the *fab* cluster of *L. lactis* MG1363 share around 70% sequence similarity with those of *S. pneumoniae*

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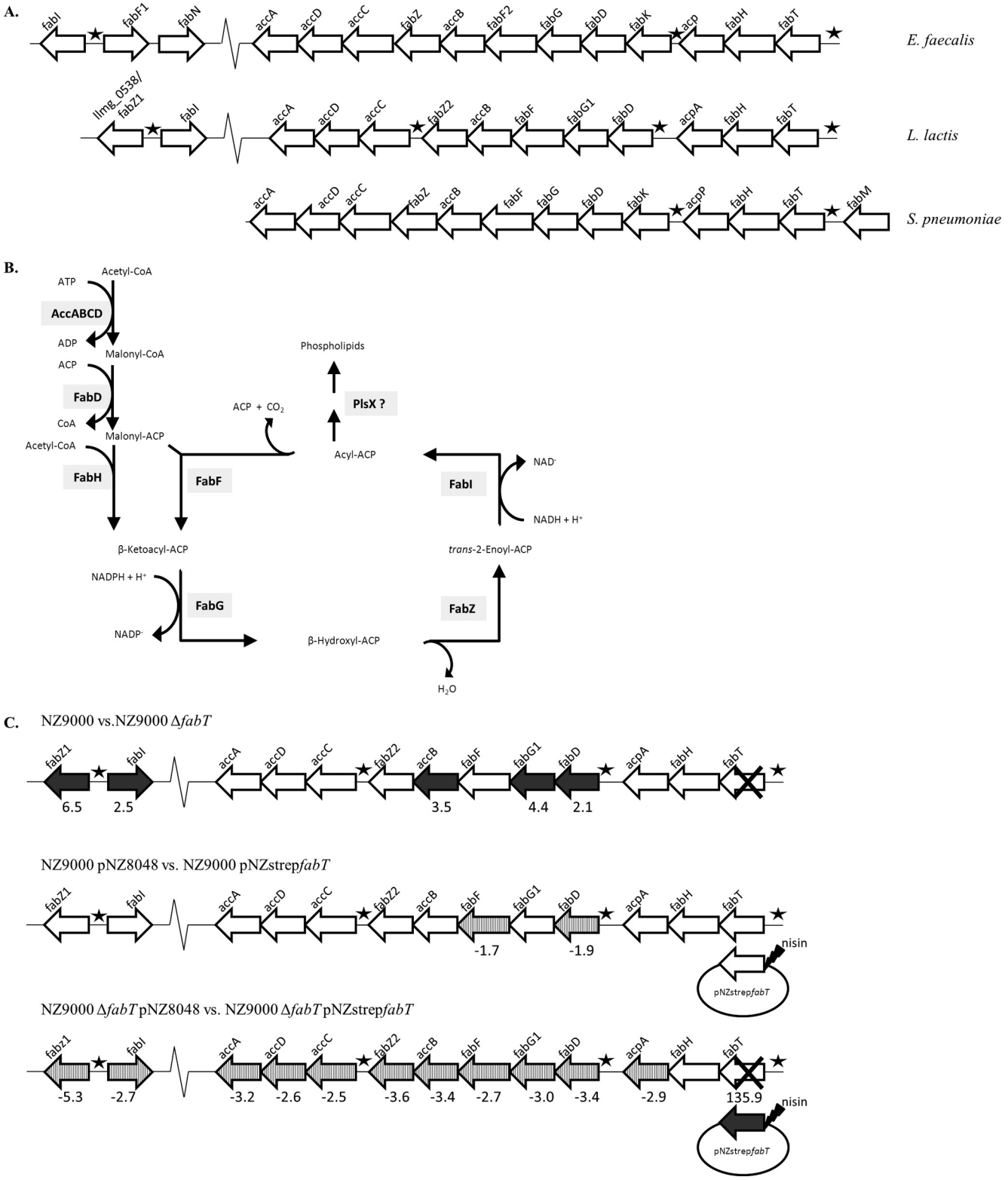

FIG 1 (A) Comparison of the *fab* clusters of *E. faecalis*, *L. lactis*, and *S. pneumoniae*. Arrows signify the *fab* genes that correspond to the open reading frames in the three organisms. Upstream regions that can bind FabT are indicated with an asterisk. (B) Schematic overview of the conserved fatty acid biosynthesis pathway, based on the annotated genes in *L. lactis* and the model of *B. subtilis* (56). AccABCD initiate the formation of malonyl-ACP. Malonyl-CoA:ACP transacylase FabD substitutes the CoA for an acyl carrier protein (ACP). Malonyl-ACP is condensed by FabH to acetyl-CoA, after which the ketoacyl-ACP is formed. β-Ketoacyl-ACP reductase FabG starts with a reduction and is followed by a dehydration executed by FabZ. A second dehydration by FabI produces acyl-ACP. Once elongated sufficiently, acyl-ACPs are processed by several unknown enzymes, the only known component being acyltransferase PlsX, and inserted into the membrane. In order to continue the elongation reaction, acyl-ACPs are dehydrated by FabF. (C) Adding or removing LlFabT has a small effect on the transcription of the FAB genes. The first microarray analysis compares the deletion of regulator FabT against the wild type. In the second analysis, induction of *fabT* on a pNZ8048 plasmid was compared against the induction of an empty pNZ8048. The bottom analysis shows the complementation of FabT, meaning the same induction of *fabT* as in the second microarray but in a *fabT* deletion background. Black genes represent upregulated genes, whereas striped genes indicate a downregulation under these conditions. Upstream regions that can bind FabT are indicated with an asterisk.

TABLE 1 Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Characteristic(s)	Reference
Strains		
L. lactis NZ9000	MG1363 derivative; *pepN*::*nisRK*; plasmid-free strain; NICE gene expression host	28
L. lactis NZ9000 Δ*fabT*	NZ9000 derivative; chromosomal deletion of *fabT*	This study
L. lactis NZ9700	Nisin-producing strain	21
E. coli DH5α	Strain carrying deletions of *recA*, *relA*, *endA*, and *hsdR17*; host of recombinant plasmids for *L. lactis*	22
Plasmids		
pCS1966	Ery^r; *oroP*; nonreplicating integration vector in *L. lactis*	20
pCS1966 Δ*fabT*	pCS1966 derivative carrying flanking regions of *fabT*	This study
pNZ8048	Chl^r; nisin-inducible gene expression vector carrying P_{nisA}	23
pNZstrep*fabT*	pNZ8048 derivative carrying Ll*fabT* with the coding region for Strep-tag at its end under the control of P_{nisA}	This study

[a] Abbreviations: *pepN*, aminopeptidase; *nisRK*, two-component system which senses extracellular nisin to activate gene transcription by P_{nisA}; *recA*, recombinational repair enzyme; *relA*, guanosine tetraphosphate (ppGpp) synthase; *endA*, endonuclease; *hsdR17*, methylation endonuclease; Ery^r, erythromycin resistance; *oroP*, orotate transporter; Chl^r, chloramphenicol resistance; P_{nisA}, nisin-inducible promoter.

D39. Although the *L. lactis*, *E. faecalis*, and *S. pneumoniae fab* gene clusters have a similar genetic organization (18), several differences remain. In this study, we establish the regulation of FA biosynthesis in *L. lactis* and compare it to that of *E. faecalis* and *S. pneumoniae*. We show that the regulator of FA biosynthesis in *L. lactis* is a repressor, which we renamed from RmaG to FabT. Moreover, we determined its regulon and its DNA binding motif by electrophoretic mobility shift assays (EMSAs) and DNase I footprinting.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *E. coli* was grown aerobically at 37°C in TY medium (1% Bacto tryptone, 0.5% Bacto yeast extract, and 1% NaCl). *L. lactis* strains were grown as standing cultures in M17 medium (Difco Laboratories, Detroit, MI) with 0.5% (wt/vol) glucose (GM17) at 30°C. Solid medium contained 1.5% agar. Chloramphenicol (5 μg/ml) and erythromycin (120 μg/ml for *E. coli* and 2.5 μg/ml for *L. lactis*) were added when required.

General DNA techniques. General molecular biology techniques were performed essentially as described by Sambrook (24). Plasmid DNA was isolated using a High Pure plasmid isolation kit and protocol (Roche Applied Science, Indianapolis, IN). Chromosomal DNA from *L. lactis* was isolated according to the method described by Johansen and Kibenich (25). PCRs for (sub)cloning were performed with Phusion (Finnzymes, Espoo, Finland) colony PCR with the *Taq* Polymerase from Fermentas (ThermoFisher Scientific Inc., Waltham, MA). Primers are listed in Table S1 in the supplemental material; they were purchased from Biolegio BV (Nijmegen, the Netherlands). PCR products were purified with a High Pure PCR product purification kit (Roche Applied Science) according to the protocol of the supplier. DNA electrophoresis was performed in 1× TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3) in 1% agarose gels with 2 μg/ml ethidium bromide. Electrotransformation was performed using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA). All DNA modification enzymes were purchased from Fermentas and used according to the manufacturer's instructions. Sequencing reactions were done at ServiceXS (Leiden, the Netherlands).

Construction of an *L. lactis fabT* deletion mutant. Upstream and downstream regions of *fabT*, PCR amplified using primer pairs Pr3/Pr4 and Pr1/Pr2 (see Table S1 in the supplemental material), respectively, were cloned in the integration vector pCS1966 (26) using the enzymes XbaI and XhoI. The resulting plasmid, pCS1966Δ*fabT*, was obtained in *E. coli* and introduced in *L. lactis* to allow integration via single-crossover homologous recombination. An *L. lactis* integrant carrying the pCS1966 construct was selected on GM17 plates with chloramphenicol. Screening for plasmid excision was done on plates containing 5-fluoroorotic acid, selecting against the *oroP* gene on pCS1966. A mutant carrying a clean knockout of *fabT* was obtained and confirmed using PCR and nucleotide sequence analysis.

***L. lactis* FabT overexpression and protein purification.** The *fabT* gene was amplified by PCR using *L. lactis* chromosomal DNA as the template and primers Pr6 and Pr61 (see Table S1 in the supplemental material). The PCR product consisting of the *fabT* gene, extended at the 5′ end with the codons for the Strep-tag (27), was purified, digested with NcoI and XbaI, and ligated into pNZ8048 (23) cut with the same enzymes. The resulting plasmid (pNZstrep*fabT*), in which the Strep-FabT construct was under the control of the nisin-inducible promoter P_{nisA}, was obtained in *E. coli* and subsequently introduced in *L. lactis* NZ9000 (28). The nucleotide sequence was confirmed by sequencing using the primers pNZSeq and nis_fw_aldert (see Table S1 in the supplemental material). Strep-tagged FabT was overexpressed using the nisin-inducible system (NICE) (23). As a source of nisin, filter-sterilized culture supernatant of the nisin-secreting strain *L. lactis* NZ9700 was used. An overnight culture of *L. lactis* NZ9000 (pNZstrep*fabT*) was diluted 100-fold in 1 liter of fresh GM17 medium with 5 μg/ml chloramphenicol and incubated at 30°C. Nisin-containing supernatant (1:500) was added when the optical density at 600 nm (OD_{600}) of the culture had reached 0.5. After 2 h of further incubation, cells were pelleted (6,000 × *g* for 10 min), resuspended in 10 ml lysis buffer (100 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 5 mg/ml lysozyme, Roche Complete Mini protease inhibitor, pH 8.0), incubated for 40 min at 30°C, and centrifuged at 9,000 × *g* (20 min, 4°C). Subsequently, 0.1 g DNase I powder was added, and the lysozyme-treated cells were broken using a French press (Aminco, Silver Springs, MD). Strep-tagged FabT was purified to homogeneity on a Strep-Tactin Sepharose column according to the manufacturer's instructions (IBA-GmbH, Göttingen, Germany). Samples from each step in the purification were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (29) and Western hybridization using anti-Strep-tag antibodies (IBA-GmbH). The concentration of purified protein was determined via spectroscopy (NanoDrop; Thermo Fisher Scientific Inc.). Protein (100 μM) was kept at −80°C in 10% glycerol, 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, pH 8.0.

DNA microarray analysis. *L. lactis* cells were grown to the mid-exponential-growth phase (OD_{600} = 0.8) in GM17 containing 5 μg/ml chloramphenicol for plasmid-containing strains. For induction of cells containing pNZstrep*fabT*, 1,000-fold dilutions of supernatant from the nisin-producing strain *L. lactis* NZ9700 were added at an OD_{600} of ~0.5. Cells were harvested by centrifugation (6,000 × *g* for 10 min); pellets were immediately frozen in liquid nitrogen and resuspended in 1.6 ml MilliQ that was treated with diethylpyrocarbonate (DEPC) (Sigma-Aldrich, St. Louis, MO) and divided in 4 portions prior to storage at −80°C. For RNA isolation, the frozen cells were thawed on ice. Subsequent cell disruption, RNA purification, reverse transcription, and Cy3/Cy5 labeling were done

as described previously (30). Labeled cDNAs were hybridized to full-genome DNA microarray slides of *L. lactis* MG1363 (31). All reagents and glassware for RNA work were treated with DEPC. RNA, cDNA quantity and quality, and the incorporation of the cyanine labels were examined by NanoDrop (Thermo Fisher Scientific Inc.) at 260 nm for RNA and cDNA, 550 nm for Cy3, and 650 nm for Cy5. Two biological replicates were used in combination with a dye swap.

DNA microarray slide images were analyzed using ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD). Filtering of bad and low-intensity spots and signals, data parsing, automated grid-based Lowess normalization, scaling, data visualization, and outlier detection were performed using the MicroPreP software (32). Differential expression tests were done on expression ratios with Cyber-T on a local server implementation of a variant of the *t* test (33). Fold changes are considered to be significant when the *P* value is ≤ 0.05 and the ratio is $\geq |1.5|$.

Fatty acid composition analysis. Samples from *L. lactis* cultures, either induced with nisin to modulate expression of Strep-FabT or not, were pelleted and washed three times in phosphate-buffered saline (PBS). All samples were transmethylated and analyzed on a gas chromatograph for acyl chain composition according to the methods described by Muskiet et al. (34). Data presented are from biological duplicates.

SDS-PAGE and Western hybridization. Protein samples were mixed (1:1) with sample buffer (120 mM Tris, pH 6.8, 50% glycerol, 100 mM dithiothreitol [DTT], 2% [wt/vol] SDS, and 0.02% [wt/vol] bromophenol blue), vortexed, boiled at 100°C for 5 min, and separated on 12% SDS-phosphonoacetic acid (PAA) gels. PageRuler prestained protein ladder (Thermo Fisher Scientific Inc.) was used as a marker. Gels were stained with Coomassie brilliant blue (0.25% Coomassie brilliant blue R-250 dissolved in 25% isopropanol with 10% acetic acid) and destained in boiling demineralized water. Western blot analysis was performed using a SNAP (Millipore Corp., Billerica, MA) system as follows: the PAA gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). Proteins were transferred to a polyvinylidene fluoride (PVDF) (Roche Applied Science) blotting membrane for 30 min at 20 V. The blot holders containing the blots were placed in the SNAP system, after which blocking buffer in PBST (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, pH 7.3, 0.1% [vol/vol] Tween 20) with 0.5% skim milk was added and the vacuum was applied. Antibodies (anti-Strep-tag conjugated to peroxidase; IBA-GmbH), diluted 1,000-fold in blocking buffer, were added to the blot holders; incubation was for 10 min at room temperature. The vacuum was removed, and the blots were washed three times with PBST prior to visualization of immunoreactive proteins using the ECL detection kit and protocol (GE Healthcare, Buckingham, United Kingdom).

Electrophoretic mobility shift assays. DNA probes comprising the upstream sequences of all genes in the *fab* operon were obtained using PCR. Probes of upstream regions of *fabI*/*fabZ1* from *L. lactis* and of *fabT* from *S. pneumoniae* were also amplified. Purified PCR products (5 μl) were end labeled with polynucleotide kinase T4-PNK (Thermo Fisher Scientific Inc.) for 2 h at 37°C by using 2 μl of 30 μCi [γ-^{33}P]ATP (PerkinElmer, Waltham, MA), 2 μl of One-For-All buffer (Roche Applied Science), and 1 μl of 10 units/μl T4-PNK in a total volume of 20 μl. End-labeled DNA was purified with a PCR purification kit (Roche Applied Science), after which the counts per minute was determined using 1 μl of each DNA sample in 4 ml of scintillation liquid (Ultima Gold; PerkinElmer).

DNA-protein binding studies were carried out in 20-μl reaction volumes containing 40 mM Tris-HCl (pH 8.0), 17.4% (vol/vol) glycerol, 2 mM EDTA (pH 8.0), 10 mM $MgCl_2$, 200 mM KCl, 1 mM dithiothreitol, labeled DNA fragment (5,000 cpm), and various amounts of purified Strep-tagged FabT protein (concentrations, 0.05 μM to 1 μM). Bovine serum albumin (2 μg) and 0.05 mg/ml poly(dI-dC) were added to the reaction mixtures in order to reduce nonspecific interactions. After incubation for 20 min at 30°C, 12 μl of the samples was loaded on a 4% PAA gel. Electrophoresis was performed in electrophoresis buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0) at 100 V for 90 min. Subsequently, the gel was dried onto Whatman 3MM filter paper, and radiolabeled bands were visualized by autoradiography using a Cyclone phosphorimager (Packard, Meriden, CT).

DNase I footprinting assay. DNase I footprinting was performed using a protocol that was largely based on the Sure Track footprinting kit (GE Healthcare), as described previously (35). The P*fabT* region of *L. lactis* was amplified by PCR using a forward primer (see Table S1 in the supplemental material) end labeled with 30 μCi [γ-^{33}P]ATP for 2 h at 37°C using T4-PNK. Binding reactions using purified Strep-FabT were identical to those used in the EMSAs, in a total volume of 40 μl and in the presence of approximately 150,000 cpm of DNA probe.

Membrane permeabilization assay. Cells were grown at 30°C to an optical density of 1.0 ± 0.1, after which 1 ml of cells was pelleted, resuspended in 100 μl PBS, and incubated with 1 μl of 1-μg/ml ethidium bromide for 15 min at 30°C. Cells were pelleted and washed two times with PBS. The cells were resuspended in 100 μl PBS and subjected for fluorescence-activated cell sorter (FACS) analysis (BD FACSCanto; BD Biosciences, Palo Alto, CA). All cells were grown as biological replicates in triplicate.

RESULTS

Two regions in the genome of *Lactococcus lactis* carry genes putatively involved in fatty acid biosynthesis (FAB) (Fig. 1A). The products of these genes show high similarities with the enzymes involved in FAB in other prokaryotes, such as *Streptococcus pneumoniae* and *Enterococcus faecalis*. Not only are the proteins conserved, the gene synteny is largely preserved (Fig. 1A). A large gene cluster extending from *llmg_1777* to *llmg_1787* contains most of the *fab* genes, while the putative *fabI* gene and a gene (*llmg_0538*/*fabZ1*) for which the product has homology with FabZ2 (*llmg_1781*) are located in a head-to-head orientation elsewhere on the lactococcal chromosome. The gene *llmg_1788* (*rmaG*) is annotated as encoding a regulator protein of the MarR family (36). This family consists of a group of dimer-forming proteins, in which both subunits possess a winged-helix DNA binding motif (37). The *rmaG* gene is located upstream of the large *fab* cluster in the *L. lactis* genome (Fig. 1A); its product shares 59% similarity with SpFabT, the transcriptional regulator of FAB in *S. pneumoniae*. Thus, Llmg1788 is hypothesized to perform an analogous function in the control of the *L. lactis fab* genes. Based on this and on the results presented in this work, we propose to rename Llmg1788 (RmaG) as *L. lactis* FabT.

FabT affects *fab* gene expression in *L. lactis*. The elimination of the *fabT* gene was achieved by complete removal of the open reading frame so that no partial *fabT* transcripts are produced in the *fabT* deletion strain. Removal of the *fabT* gene caused a slight growth delay of *L. lactis* on GM17 (data not shown), suggesting that FabT is not essential for *L. lactis* yet serves a specific function under the conditions tested. In order to determine the effects of the *fabT* mutation on gene expression in *L. lactis*, a transcriptome analysis was performed on exponential-phase cells growing in rich GM17 medium. Only a modest decrease of the *fabT* transcript was observed in the *fabT* mutant relative to its parent strain, suggesting that the quantity of *fabT* mRNA molecules is close to the background signals under these conditions in the wild-type strain. The expression of some of the genes downstream of *fabT* as well as *fabI* and *llmg_0538*/*fabZ1* is upregulated severalfold in the knockout strain (2.1- to 6.5-fold) (Fig. 1C). The transcript abundances of *acpA*, *fabHFZ2*, and *accACD* were not significantly altered.

A complementation strain which carries a copy of a gene expressing N-terminally Strep-tagged FabT, *strep-fabT*, on a plasmid downstream of the nisin-inducible promoter P_{nisA} was made.

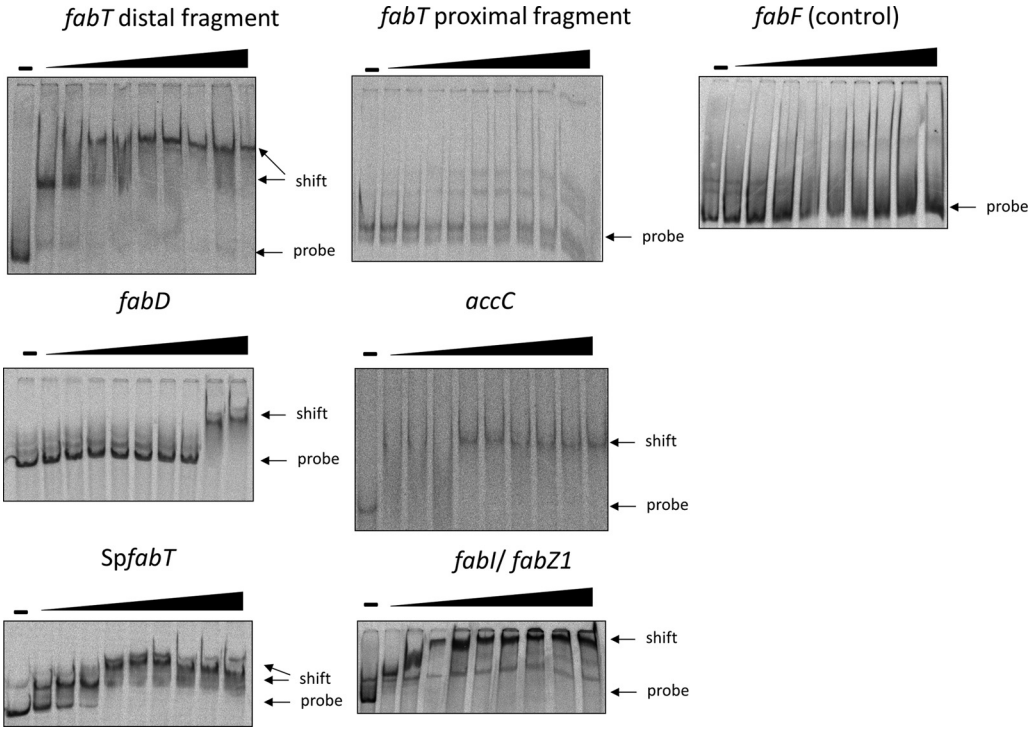

FIG 2 *In vitro* binding studies of LlFabT. Electrophoretic mobility shift assays (EMSAs) show the interaction between Strep-FabT and upstream regions of genes involved in FAB. The upstream region of *fabT* was split into two fragments, one proximal fragment (−53 bp to 189 bp upstream of the ATG) and one distal fragment (152 bp to 407 bp upstream of the ATG). The Sp*fabT* fragment is the upstream region of *S. pneumoniae fabT*, and all others are lactococcal fragments. EMSA reactions were carried out with increasing concentrations of Strep-FabT (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 1.0 μM) from left to right.

Both the wild-type strain and the *fabT* mutant strain were complemented and compared. A 136-fold upregulation of *fabT* transcripts was seen after nisin induction of *strep-fabT* (Fig. 1C). First, this shows that Strep-FabT is able to produce transcripts of *fabT* after nisin induction. Under these circumstances, a small but significant decrease in the expression of most of the *fab* genes was observed. These data support the hypothesis that FabT functions as a repressor of *fab* genes in *L. lactis*.

With the exception of *fabH*, all genes responsible for FAB are under the regulation of FabT. In addition to the *fab* cluster, transcripts of proteins with a putative or unknown function were affected. The gene *llmg_0538/fabZ1* is located next to and in opposite orientation to *fabI*; both genes thus share the same upstream region. The former is significantly downregulated (5.3-fold) when *strep-fabT* is overexpressed, while it is 6.5-fold overexpressed in the *fabT* knockout background (see Table S2 in the supplemental material).

FabT-DNA interactions. Electrophoretic mobility shift assays (EMSAs) were performed to examine whether *fab* genes are under direct or indirect control of LlFabT. Strep-FabT was overexpressed in *L. lactis* via the nisin-inducible system (NICE) (23) and purified. LlFabT runs at the position of a protein of 18 kDa, just above the band of lysozyme (14 kDa) that was used to break open the cells (see Fig. S1 in the supplemental material). A band of a protein twice the size of Strep-FabT (36 kDa) most probably represents a dimer of the regulator.

Purified Strep-FabT was incubated with the upstream DNA regions of all *fab* genes and subjected to an EMSA (Fig. 2). The results show that Strep-FabT formed protein-DNA complexes with the upstream fragments of *fabI/fabZ1*, *fabD*, *accC*, and *fabT*. In a number of cases, two shifted bands can be observed. The fact that Strep-FabT also forms a complex with a PCR fragment encompassing the Ll*fabT* promoter suggests that LlFabT regulates its own transcription. As the upstream region of Ll*fabT* used was quite large (460 bp), it was further investigated (Fig. 2). No binding to the 198-bp proximal fragment immediately upstream of the start of Ll*fabT* was observed. In contrast, significant *in vitro* binding to the more distal PCR fragment, starting 167 bp upstream of the start codon in Ll*fabT*, was detected (Fig. 2). As LlFabT and SpFabT are highly similar, binding of the former to the promoter region of *S. pneumoniae fabT* was examined. A shift was also observed when the regulator of *L. lactis* was mixed with the upstream fragment of pneumococcal *fabT* (19), containing the SpFabT binding site (Fig. 2). Binding was not seen with upstream parts of other *L. lactis fab* genes and with those of two genes of *B. subtilis* that served as negative controls (*nasD*, nitrate reductase; *htrA*, membrane-associated serine protease) (data not shown). Strep-FabT binds to all the probes in a concentration-dependent manner and with different affinities for the various fragments. The lowest concentration of protein tested (0.05 μM) was enough to cause a shift of the distal upstream region of Ll*fabT*, while at least a 0.8 μM concentration of the regulator was required to cause a shift of the upstream region of *fabD*.

DNA binding motif of lactococcal FabT. Using bioinformatic tools like MEME (38) and MDscan (39), no clear motif was able to be identified in the upstream regions bound by Strep-FabT. To pinpoint the *L. lactis* FabT DNA binding site, DNase I footprinting was performed on the distal fragment of the upstream region of

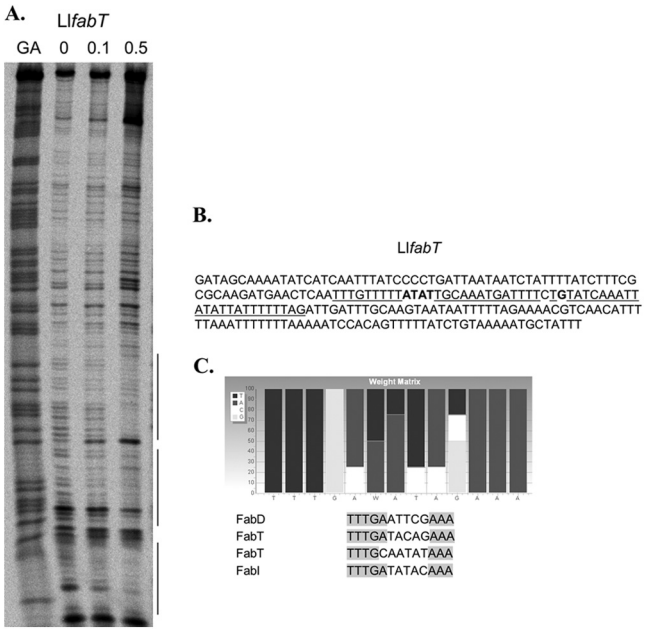

FIG 3 Purified Strep-FabT binds to its own promoter *in vitro*. (A) DNase I footprint analysis of Strep-FabT binding to the upstream region of Ll*fabT*. The first lane is the Maxam-Gilbert GA marker. The footprints are obtained by the addition of 0, 0.1, or 0.5 μM Strep-FabT protein as indicated. Black bars indicate the protected regions. (B) The sequence of the promoter region of Ll*fabT* with the protected area is underlined, and the hypersensitive base pairs are shown in bold. The transcription start site (TSS) of Ll*fabT* is indicated with double underlining. The TSS of *fabT* is at a position where the Strep-FabT protein is able to bind. (C) The weight matrix and the alignment of the four binding sites that revealed the general recognition site for FabT.

fabT. Figure 3 shows that incubation of this ^{33}P-labeled fragment with purified Strep-FabT resulted in protection of an AT-rich region of 52 bp approximately 200 bp upstream of the *fabT* start codon. Adding this information to the bioinformatics search allowed the identification of the presumed lactococcal FabT operator site (TTTGAWAWAGAAA) (Fig. 3C). This motif occurs in the upstream regions of *fabD*, *fabT*, and *fabZ1*/*fabI*. The *fabI* and *fabZ1* genes are not part of the *fab* gene cluster (Fig. 1). Nevertheless, Strep-FabT is able to form complexes with the *fabI*/*fabZ1* intergenic region (Fig. 2). The transcription start sites (TSS) of both *fabI* and *fabZ1* were determined via 5′ rapid amplification of cDNA ends (RACE) (Fig. 4B). The DNA binding motif overlaps the TSS of both *fabZ1* and *fabI*; thus, FabT may block the transcription of both genes simultaneously.

Effect of FabT on *L. lactis* membrane phospholipid composition. *L. lactis* FabT controls the transcription of most *fab* genes, the encoded proteins of which produce acyl chains that are incorporated into phospholipids. Consequently, the effect of *fabT* deletion or overexpression on the fatty acid composition of the cytoplasmic membrane phospholipids was examined by gas chromatography. Overproduction of Strep-FabT in both the wild-type strain of *L. lactis* and the *L. lactis fabT* mutant does not affect the length of the fatty acids or their degree of saturation (Fig. 5). Removal of FabT results in a shift from unsaturated (18:1n7) to saturated (16:0 and 18:0) fatty acids. Approximately 50% of the acyl chains in the *fabT* mutant are 16:0 molecules. The relative amount of 18:0 molecules is at least three times higher in the *fabT* knockout mutant than in the parent strain. The main decrease is observed in oleic acid (18:1n9), an unsaturated fatty acid. This shift in the UFA/SFA ratio had an effect on the membrane permeability. The *fabT* mutant, possessing higher relative amounts of saturated fatty acids, had a higher membrane permeability for the fluorescent DNA binding probe ethidium bromide (Fig. 5B).

DISCUSSION

Elucidating the regulation of the essential pathway of fatty acid biosynthesis in the industrially relevant bacterium *L. lactis* is of interest from both fundamental and application points of view. Here, we characterized the regulation of FAB in *L. lactis*. The gene for the MarR-type regulator RmaG, here renamed FabT, is located in a gene cluster containing most of the *L. lactis fab* genes. The genetic makeup of the gene cluster and the synteny and orientation of the *fab* genes are very similar to those of the *fab* clusters of *S. pneumoniae* and *E. faecalis* (40, 41). A transcriptome analysis of the effect of deleting the *fabT* gene from the chromosome of *L. lactis* clearly revealed that FabT is involved in the regulation of the *fab* gene cluster and in *fabZ1*/*fabI* expression. No other genes were significantly regulated by FabT, indicating that it is a dedicated

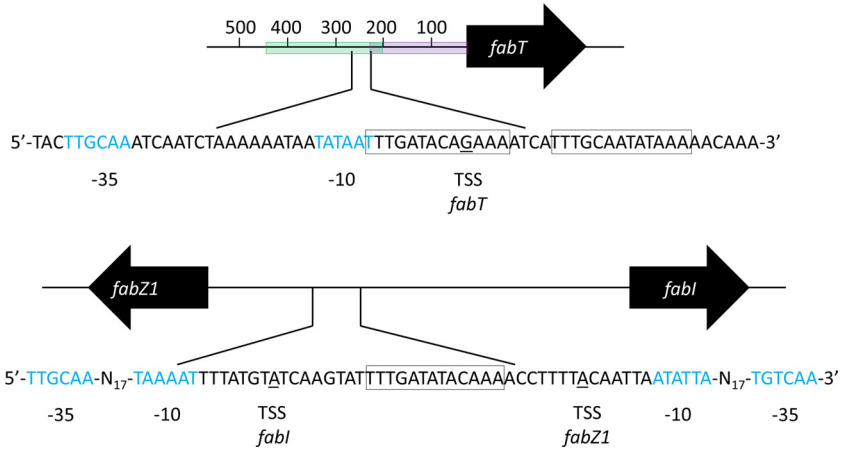

FIG 4 Transcription start sites of *fabT* (A) and *fabZ1* and *fabI* (B). Transcription start sites were determined with 5′ RACE (underlined). Nucleic acid bases in blue are the −10 and −35 sites. The PCR fragments used for EMSAs are named FabT proximal fragment (purple) and FabT distal fragment (green). The binding motif of Strep-FabT is indicated with a box. Upon FabT binding, transcription of *fabI* and *fabZ1* was able to be blocked simultaneously.

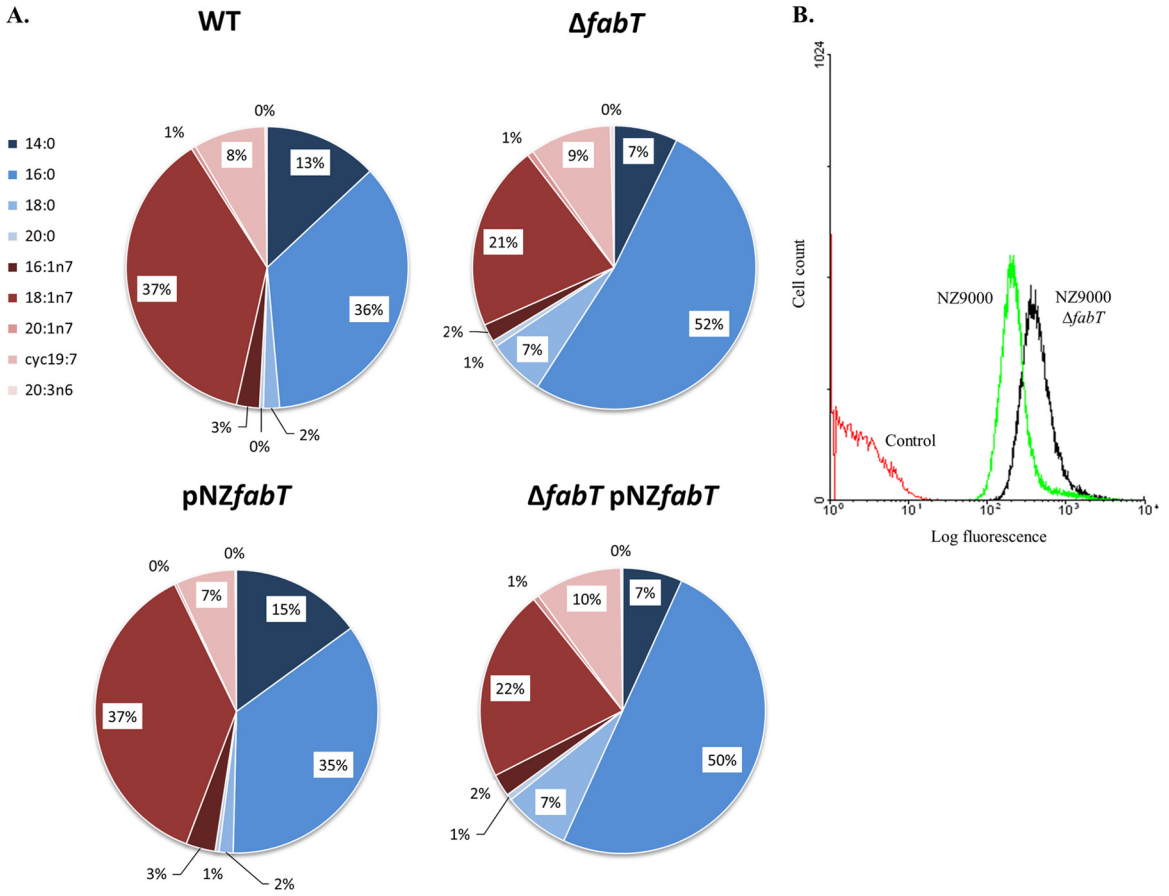

FIG 5 (A) Fatty acid composition of *L. lactis* strains. Deleting FabT (Δ*fabT*) creates a shift from unsaturated (18:1n7) to saturated (16:0 and 18:0) acyl chains. Induction of *fabT* (pNZ*fabT*) does not affect fatty acid composition. Cyc19:7 is a cyclic fatty acid, and saturated acyl chains are shown in blue, while unsaturated acyl chains are shown in red. Shown are the averages of two biological experiments. (B) Effect of the *fabT* mutant on membrane permeability to ethidium bromide. The control (red line) is the NZ9000 strain without exposure to fluorescent ethidium bromide, while NZ9000 (green line) and NZ9000 Δ*fabT* (black line) were both incubated with 1% (vol/vol) of 1-μg/ml ethidium bromide for 15 min and subsequently washed to remove extracellular ethidium bromide. The graphs shown are representative of two independent biological experiments performed with triplicates.

local regulator of FAB. Upon overexpression of *strep-fabT*, only *fabF* and *fabD* respond, with significant downregulation of −1.7- and −1.9-fold, respectively. The repressive capacities of FabT were more apparent when its gene was removed from the genome. When the *fabT* mutant was complemented by overexpression of Strep-FabT, almost the complete *fab* cluster responded (Fig. 1C). The only gene in the *fab* operon that does not seem to respond to the deletion or overexpression of FabT is *fabH*. It was previously reported that an *L. lactis* β-ketoacyl-ACP synthase III (FabH) mutant strain survives (42). In that case, β-ketoacyl-ACP synthase I (FabF) took over the condensation function of the deleted *fabH*. Similarly, *S. pneumoniae fabM* transcription does not respond to the deletion of the streptococcal *fab* regulator FabT (40). Isomerase FabM was described not to be essential in this organism (5). In *Streptococcus mutans*, FabM is essential at low pH (43). FabH of *L. lactis* seems to be nonessential, as its transcription does not respond to deletion of *fabT* or to the overexpression of Strep-FabT. Altogether, it seems that noncrucial *fab* genes do not directly respond to the presence or absence of the *L. lactis* FAB regulator.

Under the conditions employed, exponential growth in a rich medium, it seems that the repressive effect of *L. lactis* FabT on *fab* gene expression is mild; only delicately repressing the genes involved in FAB appears to be sufficient to control regulation. A mild repression of the FAB enzyme genes that can easily be relieved may therefore be sufficient for cell maintenance. The DNA microarrays report that significant changes occur in transcription of the *fab* cluster. FabT does not regulate genes for membrane proteins like PlsX and PlsY, which are involved in glycerolipid formation in *B. subtilis* (11), or cyclic fatty acid synthase Cfa (44). Thus, *L. lactis* FabT seems to be a dedicated repressor: it regulates only fatty acid biosynthesis.

In *E. coli*, the regulator FadR has a dual function of stimulating biosynthesis and limiting the degradation pathway of FA (45). Such a dual function of FabT of *L. lactis* or *S. pneumoniae* is difficult to examine at this point, as in these bacteria, fatty acid degradation has not yet been characterized and the genes have not been identified. The fact that the regulon of *L. lactis* FabT seems to be strictly confined to the FA biosynthesis genes suggests that in this organism, FA degradation is controlled by a different mechanism. Investigating a range of different candidates for binding to *S. pneumoniae* FabT shows that acyl-ACPs of appropriate length (C16:0/C18:0) are the best ligands (19). It seems reasonable that for *Streptococcaceae*, transcription of the FAB genes is low but constitutive and is diminished

even more when more unsaturated acyl-chains are present than are needed.

The proposed binding motif of *S. pneumoniae* FabT is GTTTT GATTGTAAAAGT (40), while the consensus binding motif of FabT in *E. faecalis* was proposed as AGTTTGATAATCAAATT (41). Using these motifs did not automatically reveal the binding site for *L. lactis* FabT. Ultimately, the consensus binding motif of *L. lactis* FabT was determined to be **TTTGA**WAWA**GAAA** (boldface indicates fully conserved residues). The lactococcal FabT binding motif displays a lot of variation, with a bias toward adenines in the 3′ end of the motif and a distinct TTTGA in the 5′ end, as is also the case in the binding sites of the FabT regulators of the other two bacterial species. The similarity of the FabT binding sites is high enough for *L. lactis* FabT to recognize and bind to the *S. pneumoniae* FabT binding site (Fig. 2). It is therefore highly likely that the presence of saturated fatty acids as corepressors will affect the binding affinity, as has been shown for *S. pneumoniae* (19, 40), but not the nature of the recognition sequence. While a lot of MarR regulators prefer to bind inverted repeats, not all members of the helix-turn-helix family do so (46, 47). Also, here, a clear palindrome or repeat cannot be seen in the FabT binding motifs in *L. lactis*, *S. pneumoniae*, and *E. faecalis* except the outermost triple Ts and triple As. Apparently, the minimal common motif of FabT in *Streptococcaceae* does not require a palindromic motif. Whereas FabT of *S. pneumoniae* binds only to the promoter region of its own gene and to that of *fabK*, *L. lactis* FabT binds to the upstream regions of its own gene, *fabT*, and, further downstream in *fab*, to the upstream regions of *fabD*, *accC*, and *fabI* (Fig. 1C).

In order to adapt to changing environments, membrane fluidity and permeability in bacteria are changed by altering acyl chain composition and head group modification. It was suggested that acyl chain composition is modified mainly by *de novo* synthesis (48). *L. lactis* possesses all enzymes necessary to produce saturated acyl chains. However, for the production of unsaturated acyl chains, no equivalent of a desaturase like *B. subtilis* DesK (49) is annotated in the genome of *L. lactis*. *E. coli* uses the dehydratase FabA and the condensation enzyme FabB (10) to produce *cis* double bonds, while two dehydratase FabZ variants exist in *E. faecalis*, of which one (FabN) can function as an isomerase to create unsaturated fatty acids (41). The *S. pneumoniae cis-trans* isomerase FabM functions as an interconverter in competition with enoyl-ACP reductase FabK: the 10:1-*trans*-2 intermediate is either processed into saturated fatty acids or altered via FabM into a 10:1-*cis*-3 intermediate that is further converted into other unsaturated fatty acids (40). If *L. lactis* produces unsaturated fatty acids according to the models of FAB of *E. coli* or *S. pneumoniae*, the corresponding enzymes still need to be identified. It seems more likely that *L. lactis* uses a mechanism similar to that operative in *E. faecalis*, as its genome contains two genes, *fabZ1* and *fabZ2*, of which the encoded enzymes are similar (75% and 69%) to FabN and FabZ of *E. faecalis* V583, respectively. Also, *L. lactis fabZ1* is in close proximity to *fabI*, as is the case for *E. faecalis fabN/fabI* (Fig. 1A). Even though *fabZ1*/*fabI* are on a different location in the chromosome, the *L. lactis* FabT binding motif is present in their intergenic region. The 5′ RACE and microarray results further support the idea that *fabZ1* is under the direct regulation of FabT in *L. lactis*.

The repressor FabT has a direct effect on transcription of most of the FAB genes. To determine the physiological effects of the *fabT* mutant, the acyl chain composition was determined. Deletion of *fabT* in *L. lactis* leads to an increase in SFA from 51% to 66% (Fig. 5). Complementation of the mutation by overexpression of the Strep-FabT repressor, even for as long as 2 h (approximately two doubling times), does not significantly change the UFA/SFA ratio. Apparently, two generations of repressive circumstances on the genes in the *fab* operon do not deplete the already available FAB enzymes to such an extent as to have an effect on this ratio. Alternatively, the high intracellular amounts of Strep-FabT, in the absence of corepressor molecules due to the observed repression of the *acp* gene, might result in such a disturbed FAB regulation that the wild-type UFA/SFA ratio of 50% can no longer be obtained.

A change in membrane fluidity due to a change in the UFA/SFA ratio can be observed by an increased uptake of ethidium bromide (50). Higher relative amounts of saturated fatty acids in the *L. lactis fabT* mutant result in a higher permeability for a compound such as ethidium bromide (Fig. 5B). This increased sensitivity for the uptake of such compounds may potentially be an interesting phenotype for industrial applications. Engineering FAB for industrial purposes has shown to be effective for *E. coli*, indicating the robustness of this synthetic route (51).

In studies in which various stress conditions have been applied to *L. lactis*, changes in expression of the genes for fatty acid biosynthesis are often seen (52–55). Further characterization of the *fab* cluster and the regulatory mechanism identified here will help to understand the intricacies of membrane integrity and modulation in *L. lactis*.

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